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STUDIES ON THE PURIFICATION OF VACCINIA VIRUS

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STUDIES ON THE PURIFICATION OF VACCINIA VIRUS

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ABSTRACT

Various methods are described and compared for removing extraneous matter from vaccinia virus suspensions. Of ten procedures tested, differential centrifugation and Freon extraction were the most successful. Both the partially purified suspensions and the dried products prepared from them had high virus titers in embryonated eggs and high infectivity for mice. Although both processes resulted in products with the same titers, the differentially centrifuged product was approximately tenfold more purified as shown by the percentage solids of the suspensions before freeze drying.

I. INTRODUCTION

Recent literature shows that living attenuated vaccines are rapidly replacing killed vaccines. Sabin,[1] Zhdanov,[2] and Smorodinstev[3] have demonstrated the superiority of living over killed vaccines. Burnet[4] emphasized, however, that the antigenic potency of living vaccines must be maintained. This can be accomplished by preservation of the viability of the microorganisms by freeze drying as reported by Cross et al.[5] However, an important aspect of preparing dried vaccine is purification of the virus suspensions prior to freeze drying. Kim,[6] Gessler et al,[7,8] and Epstein[9] have successfully purified vaccinia virus suspensions by the fluorocarbon technique; Cross et al[5] utilized differential centrifugation. The extent of purification was determined by: (a) titration in embryonated eggs, (b) vaccination, (c) electron microscopy, or (d) combinations of these.

The purification techniques for vaccinia virus were not compared, nor did the investigators report the effects of the purification technique upon the animal infectivity of the virus. Riley and Orlando[10] have stressed the need for assaying purified preparations in both eggs and mice because infectivity may vary for different hosts.

In this study two basic methods of purification and combinations of these methods are compared without final measurement of antigenic properties. The efficiency of the purification techniques was evaluated by: (a) the amount of extraneous matter removed, (b) infectivity based upon virus titers in embryonated eggs, (c) infectivity for animals,* and (d) the potency of the dried product as measured by virus titers and animal infectivity without measurement of antigenic properties.

* In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

II. MATERIALS AND METHODS

A. VIRAL SUSPENSIONS

Vaccinia virus (International Health Division strain) was used in this study. Eleven-day-old embryonated eggs were inoculated on dropped chorio-allantoic membranes (CAM) as described by Hahon *et al.*[11] The CAM were harvested after incubation at 35°C for 72 hours.

B. PURIFICATION PROCEDURES

1. Differential Centrifugation

An 80 per cent suspension of CAM was prepared in sterile distilled water, blended* for two minutes, and clarified by centrifugation for five minutes at 1000g to remove gross particulates. The virus in the supernatant was concentrated in a Sharples laboratory model centrifuge at 52,000g in a batch bowl. The sediment was resuspended in sterile distilled water.

2. Solvent Extraction

a. Freon Extraction

A 50 per cent suspension of CAM was prepared in sterile distilled water, blended for two minutes, and centrifuged for five minutes at 1000g to remove gross particulates. The supernatant was mixed with Freon-113 in a 1:1 ratio, blended for two minutes, and centrifuged for three minutes at 23,500g. This centrifugation produced a triple-layered product as follows: (a) a bottom layer of Freon-113, (b) a middle layer of fatty residue, and (c) a top aqueous layer containing the partially purified virus.

b. Acetone Extraction

The CAM harvest was mixed with acetone in a 1:10 ratio, blended for two minutes, and partially purified by passage through a coarse fritted glass filter. The concentrated mixture was centrifuged for three minutes at 23,500g. The sediment was reprocessed until four acetone extractions had been completed.

c. Ether Extraction

The CAM harvest was mixed with ether in a 1:10 ratio and processed as in Product 2, b above.

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* Servall Omni-mixer, Ivan Sorvall, Inc., Norwalk, Connecticut.

3. Combinations

a. Combination of Differential Centrifugation and Ether Extraction

An 80 per cent suspension of CAM was treated as in Product 1. One part of the sediment was mixed with two parts of ether, blended for two minutes, and centrifuged for three minutes at 23,500g. This procedure was repeated twice to give a total of three ether extractions.

b. Combination of Differential Centrifugation and Acetone Extraction

An 80 per cent suspension of CAM was treated as in Product 1. The sediment was mixed with acetone in a 1:1 ratio, blended for two minutes, and centrifuged for three minutes at 23,500g. This procedure was repeated twice to give a total of three acetone extractions.

c. Combination of Freon Extraction and Acetone Extraction

A 50 per cent suspension of CAM was treated as in Product 2,a. The aqueous layer was mixed with acetone in a 1:1 ratio, blended for two minutes, and centrifuged for three minutes at 23,500g. The sediment was mixed with acetone in a 1:1 ratio, blended, and passed through a coarse fritted glass filter. The concentrated virus was resuspended in sterile distilled water.

d. Combination of Freon Extraction and Acetone Extraction

A 50 per cent suspension of CAM was treated as in Product 3,c without filtration through the fritted glass filter.

e. Combination of Freon Extraction and Ether Extraction

A 50 per cent suspension of CAM was treated as in Product 2,a. One part of the aqueous layer was mixed with two parts of ether, blended for two minutes, and centrifuged for three minutes at 23,500g. The concentrated vaccinia was resuspended in sterile distilled water.

f. Combination of Acetone Extraction and Freon Extraction

The CAM harvest was mixed with acetone in a 1:2.5 ratio, blended for two minutes, and centrifuged for five minutes at 1000g. The sediment was resuspended in sterile distilled water and mixed with Freon-113 in a 1:1 ratio. The mixture was blended for two minutes and centrifuged for three minutes at 23,500g. The top aqueous layer contained the partially purified virus.

4. Control

A 20 per cent suspension of CAM was prepared in heart infusion broth (HIB).

C. DRYING

Products 2,b, 2,c, 3,a, and 3,b were dried *in vacuo*; all others were freeze dried.

D. SOLIDS

The per cent solids of the liquid slurries were determined on a Cenco Moisture Balance.

E. ASSAYS

Pock counts were made in the usual manner by inoculation of 0.1 milliliter of appropriate dilutions on the dropped CAM of eleven-day-old embryonated eggs. Ten eggs were inoculated for each dilution and pock-forming units (PFU) were counted after 72 hours of incubation at 35°C.

For LD_{50}'s, groups of ten mice, Swiss Webster strain, weighing 10 to 14 grams were inoculated intracerebrally with 0.03 milliliter of serial dilutions. The mice were observed for 14 days and deaths were recorded daily. The LD_{50} value was calculated by the Reed-Muench method.[12]

III. RESULTS AND DISCUSSION

The efficiency of the purification techniques was evaluated by four criteria: (a) removal of extraneous matter as measured by the percentage solids of the liquid slurry; (b) infectivity based upon the virus titers in embryonated eggs; (c) infectivity based upon animals as demonstrated by intracerebral inoculation of mice; and (d) the potency of the dried product by measurement of the virus titers and animal infectivity.

The percentage solids of the harvested CAM before treatment was 7.5 per cent. As shown in Table I, the efficiency of seven of the purification processes can be evaluated readily by the percentage solids of the liquid slurries. The purification efficiencies of the differential centrifugation process (Product 1) and of the combination process of acetone extraction followed by Freon extraction (Product 3,f) were significantly better than those of other treatments. The differential centrifugation process removed 95 per cent and the combination of acetone and Freon extraction 88 per cent of the extraneous material. These two processes were at least four times more efficient for purification of the CAM harvest than the next best treatment. The other four purification processes could not be evaluated on the basis of percentage solids because those treatments produced partially dried materials that were subsequently dried *in vacuo*.

An examination of the virus titers of the liquid slurries (Table II) shows an increase in titer for five of the six purification processes. The increases ranged from one to five log units, and the greatest increase in titer is associated with Freon extraction (Product 2,a). This remarkably large increase in titer demonstrates that Freon not only partially purified the CAM harvest (9.33 per cent as shown by percentage solids) but may also have liberated virus particles that had not been released by blending. Differential centrifugation (Product 1) was associated with a two-log increase in virus titer. In contrast to the increase in virus titers of five purification processes, the purification process involving Freon extraction followed by ether extraction (Product 3,e) apparently resulted in destruction of the virus, as evidenced by the absence of growth on the CAM and the loss of animal infectivity.

Our third criterion, animal infectivity of the liquid slurries, eliminated four of six possible purification processes. Only two treatments, Freon extraction (Product 2,a) and differential centrifugation (Product 1), were associated with animal infectivity titers equal to the control (Product 4).

TABLE I. EFFICIENCY OF PURIFICATION PROCESS FOR VACCINIA VIRUS AS MEASURED BY PERCENTAGE SOLIDS

Purification Process	Solids, %		Solids Removed by Purification, %	Purification Efficiency, %
	Before Processing	After Processing		
1. Differential centrifugation	6.00	0.30	5.70	95.00
2. Solvent extraction				
a. Freon extraction	3.75	3.40	0.35	9.33
3. Combinations				
c. Freon, acetone extraction (filtration)	3.40	3.00	0.40	11.76
d. Freon, acetone extraction (no filtration)	3.75	3.00	0.75	20.00
e. Freon, ether extraction	3.75[a]	3.00	0.75	20.00
f. Acetone, Freon extraction	7.50	0.90	6.60	88.00
4. Control[b]	5.00			

a. After Freon extraction, 3.40.
b. Twenty per cent CAM in heart infusion broth.

TABLE II. BIOLOGICAL CHARACTERISTICS OF PURIFIED VACCINIA VIRUS BEFORE AND AFTER FREEZE DRYING

Purification Process	Liquid Preparation		Dried Materials	
	PFU[a]/ml	$MICLD_{50}$/ml[b]	PFU/gm	$MICLD_{50}$/gm[b]
1. Differential centrifugation	8.05×10^{10}	8.88	2.03×10^{13}	8.77
2. Solvent extraction				
a. Freon	1.65×10^{13}	7.28	6.50×10^{12}	8.70
b. Acetone	NA[c]	NA	3.76×10^{8}	5.50
c. Ether	NA	NA	[d,e]	[f]
3. Combinations				
a. Differential centrifugation, ether	NA	NA	[d,e]	[f]
b. Differential centrifugation, acetone	NA	NA	1.01×10^{13}	6.14
c. Freon, acetone, filtered	1.14×10^{9}	[g]	1.28×10^{13}	[f]
d. Freon, acetone, not filtered	1.40×10^{9}	3.28	0.98×10^{8}	7.07
e. Freon, ether	[d]	[g]	[d,e]	[f]
f. Acetone, Freon	2.80×10^{11}	[g]	1.50×10^{13}	5.76
4. Control[h]	1.50×10^{8}	7.83	1.49×10^{13}	8.68

a. Pock-forming units.
b. Mouse intracerebral lethal dose, 50 (to base 10) per milliliter or gram.
c. Not applicable.
d. Assays repeated six times; no growth recorded.
e. Very coarse, oily mass; not a dried product; discarded.
f. No deaths when 0.03 ml of 10^{-4} dilution was injected intracerebrally (IC).
g. No deaths when 0.03 ml of 10^{-1} dilution was injected IC.
h. Twenty per cent CAM in heart infusion broth.

The final criterion for evaluation of the purification processes was the potency of the dried product as measured by the virus titers in embryonated eggs and animal infectivity. The assays of the dried materials obtained with the control treatment (Product 4) demonstrated high virus titers (1.49×10^{13} per gram) and animal infectivity ($\log_{10}$ $MICLD_{50}$/grams, 8.68). The high virus titer of 1.49×10^{13} per gram cannot be accounted for by water removal because, on the basis of per cent solids of the suspension before freeze drying, a pock count of 3×10^{10} per gram would represent a 100 per cent recovery. We can only theorize that blending the suspension before freeze drying does not completely disrupt the pocks into individual particles, although the stresses of freeze drying do disrupt the larger aggregates into smaller particles.

Two purification processes, Freon extraction (Product 2,a) and differential centrifugation (Product 1), were associated with equally high titers, but all other treatments were associated with significantly lower virus titers, significantly decreased animal infectivity, or both. The high titers obtained by Freon extraction and differential centrifugation are exceptionally striking, since the stabilizing influence of additives was not used for these treatments. These titers compare favorably with those of the controls, which had the excellent protective qualities of heart infusion broth for freeze drying.

The purification processes that used either acetone or ether extraction produced disappointing results. The use of acetone extraction in the purification process significantly decreased the animal infectivity in all treatments, and in one treatment (Product 3,c) the virus had lost its lethality for mice. This was especially discouraging because three products (3,b, 3,c, and 3,f) had pock titers equal to the control. Ether extraction produced in all cases a very coarse, oily mass that apparently did not contain any viable particles, as shown by inoculation of the CAM and intracerebral inoculation of mice.

IV. CONCLUSIONS

Differential centrifugation and Freon extraction were used successfully for the partial purification and subsequent freeze drying of vaccinia virus. Although both processes result in products with the same titers (virus and animal infectivity), the differentially centrifuged product is approximately tenfold more purified as shown by the percentage solids of the suspensions before freeze drying.

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